Development of epoxyeicosatrienoic acid analogs with *in vivo* anti-hypertensive actions

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Epoxyeicosatrienoic acids (EETs) contribute importantly to the regulation of vascular tone and blood pressure control. The purpose of this study was to develop stable EET analogs and test their *in vivo* blood pressure lowering effects in hypertensive rats. Using the pharmacophoric moiety of EETs, ether EET analogs were designed with improved solubility and resistance to auto-oxidation and metabolism by soluble epoxide hydrolase. Ether EET analogs were chosen based on their ability to dilate afferent arterioles and subsequently tested for blood pressure lowering effects in rodent models of hypertension. Initially, 11,12-ether-EET-8-ZE failed to lower blood pressure in angiotensin hypertension or spontaneously hypertensive rats (SHR). Esterification of the carboxylic group of 11,12-ether-EET-8-ZE prevented blood pressure increase in SHR when injected at 2 mg/day for 12 days (MAP Δ change at day 8 of injection was -0.3 ± 2 for treated and 12 ± 1 mmHg for control SHR). Amidation of the carboxylic group with aspartic acid produced another EET analog (NUDSA) with a blood pressure lowering effect when injected at 3 mg/day in SHR for 5 days. Amidation of the carboxylic group with lysine amino acid produced another analog with minimal blood pressure lowering effect. These data suggest that esterification of the carboxylic group of 11,12-ether-EET-8-ZE produced the most effective ether-EET analog in lowering blood pressure in SHR and provide the first evidence to support the use of EET analogs in treatment of cardiovascular diseases.

Keywords: hypertension, blood pressure, afferent arteriole, epoxyeicosanoids, vasodilation

INTRODUCTION

Epoxyeicosatrienoic acids (EETs) are synthesized from arachidonic acid by epoxygenase enzymes. These EETs have biological actions that suggest that EETs would be beneficial in combating cardiovascular diseases (Zeldin, 2001; Spector et al., 2004; Imig, 2005; Fleming, 2007). Cardiovascular actions described for EETs include identification as endothelium-derived hyperpolarizing factors, inhibition of leukocyte adhesion to the vascular wall, inhibit vascular smooth muscle migration, promote endothelial cell proliferation, and inhibit platelet aggregation (Campbell et al., 1996; Fisslthaler et al., 1999; Node et al., 1999; Sun et al., 2002; Potente et al., 2003; Krotz et al., 2004; Hercule et al., 2009). EETs have also been demonstrated to preserve organ function when added to a transplantation preservation solution (Yang et al., 2003). Myocardial infarct size following ischemia reperfusion is decreased by the presence of EETs in the reperfusion solution (Seubert et al., 2007; Gross et al., 2008). However, EET or EET analogs have been used with limited success when administered *in vivo* either acutely or chronically. Given the cardiovascular actions attributed to EETs it has been postulated that modulation of EETs in cardiovascular diseases has potential therapeutic value.

One approach to target EETs for cardiovascular diseases is the development of agonistic analogs for the EETs. EET analogs were developed for *in vitro* studies because of the limited solubility and storage issues with endogenous EETs (Imig et al., 1999; Falck et al., 2003a). These EET analogs were designed to resist metabolism and improve solubility and facilitated the identification of structure activity relationships for 11,12-EET and 14,15-EET (Falck et al., 2003a; Dimitropoulou et al., 2007; Yang et al., 2007; Falck et al., 2009). EET analogs vasodilate coronary, cerebral, renal and mesenteric arteries, as well as, inhibit vascular smooth muscle cell tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression (Falck et al., 2003b; Gauthier et al., 2004; Falck et al., 2009; Sudhahar et al., 2010). Evidence has also supported the use of EET analogs in cardiovascular disease. The sulfonimide analog of 11,12-EET (11,12-EET-SI) used *in vitro* improved vascular function in afferent arterioles taken from hypertensive rats (Imig et al., 2001). EET analogs also decrease heart damage in animal models of cardiac reperfusion injury (Seubert et al., 2007; Gross et al., 2008). Recently 11,12-EET analogs based on the 11-nonyloxy-undec-8-(*Z*)-enoic acid structure have been generated with better solubility and resistance to β-oxidation (Imig and Falck, 2009; Sodhi et al., 2009). One promising EET analog that has been used successfully when administered *in vivo* is the 11,12-EET analog, NUDSA, that is the aspartic amide of 11-nonyloxy-undec-8(*Z*)-enoic acid (Sodhi et al., 2009). The purpose of the current study was to develop EET analogs and test their *in vivo* potential to lower blood pressure in rats with hypertension.

MATERIALS AND METHODS

EET ANALOG DESIGN AND SYNTHESIS

11,12-EET analogs were synthesized for use in experimental protocols (**Figure 1**). The synthesis for the following EET analogs have been previously described; 11,12-ether-EET-8-ZE (Falck et al., 2003b), EET-NOX-8-glyceride (Imig and Falck, 2008a), NUSGLY (Imig and Falck, 2008a), 14,15-Ether-EEZE (Imig and Falck, 2008a), EET-NOX-8-sulfonate (Imig and Falck, 2008b), NUDSA (Sodhi et al., 2009), EET-NOX-8-mann (Imig and Falck, 2008b), and EET-NOX-PEG (Imig and Falck, 2008b).

SYNTHESIS OF (S)-7-AMINO-3-[11-(NONYLOXY)UNDEC-8(Z)-ENAMIDO] HEPTANOIC ACID HYDROCHLORIDE (NUSLY)

2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 224 mg, 0.58 mmol), N_e-Boc-L-lysine methyl ester hydrochloride (153 mg, 0.58 mmol), and diisopropylethylamine (205 μL) were added sequentially to a stirring, room temperature solution of 11-(nonyloxy)undec-8(Z)-enoic acid (170 mg, 0.52 mmol) in anhydrous DMF (5 mL) under an argon atmosphere. After stirring 12 h, the reaction mixture was diluted with EtOAc (100 mL) and thoroughly washed with water (3 × 100 mL), brine (2 × 100 mL), dried over Na_2SO_4, and evaporated in *vacuo*. The residue was purified by SiO_2 column chromatography eluting with 50% EtOAc/hexane to afford methyl (S)-7-(*tert*-butoxycarbonylamino)-3-(11-(nonyloxy)undec-8(Z)-enamido)heptanoate (201 mg, 68% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$ 300 MHz) δ 6.07 (d, J = 6.9 Hz, 1H), 5.44–5.32 (m, 2H), 4.61–4.54 (m, 2H), 3.72 (s, 3H), 3.41–3.55 (m, 4H), 3.12–3.04 (m, 2H), 2.30 (q, J = 6.9 Hz, 2H), 2.20 (t, J = 7.8 Hz, 2H), 2.02–2.00 (m, 2H), 1.64–1.42 (m, 16H), 1.29–1.24 (m, 20H), 0.85 (t, J = 6.6 Hz, 3H).

To a stirring, 0°C solution of the above ester (200 mg, 0.35 mm) in THF/H_2O (4:1, 5 mL) was added LiOH (~2 mL, 1 M aqueous solution). After stirring 12 h at room temperature, the reaction

FIGURE 1 | Chemical composition of epoxyeicosatrienoic acid (EET) analogs.
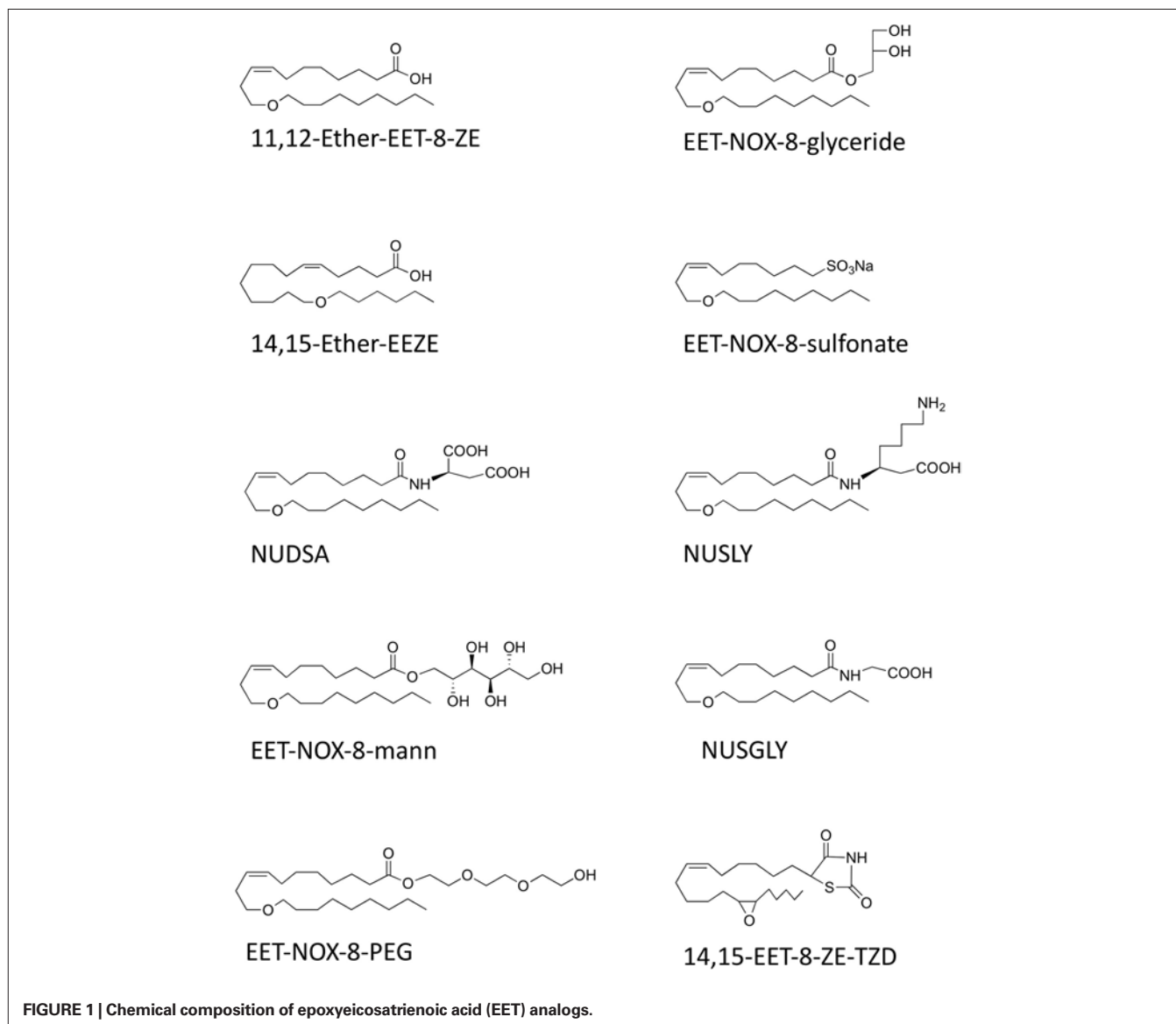
mixture was cooled to 0ºC, acidified to pH ~ 4.5 using oxalic acid (~1 mL, 1 M aqueous solution), and extracted with EtOAc (2 × 50 mL). The combined organic extracts were washed with water (2 × 100 mL), brine (1 × 100 mL), and dried over Na_2SO_4. The solvent was removed in *vacuo* and the residue was subjected to Boc-deprotection in THF/H_2O (1:1, 5 mL) saturated with HCl gas. The solvent was evaporated, and dried over high vacuum for 2 h to afford the title compound (65 mg, 55% yield). ^{1}H NMR (MeOH-d$_4$, 300 MHz) δ 5.44–5.32 (m, 2H), 4.42–4.34 (m, 1H), 3.41–3.55 (m, 4H), 2.95–2.82 (m, 4H), 2.29–2.23 (m, 4H), 2.12–1.41 (m, 14H), 1.37–1.28 (m, 16H), 0.86 (t, $J = 4.8$ Hz, 3H).

ANIMALS

Experiments were conducted using male rats and animal protocols were in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committees. Rats were fed a normal rat chow diet throughout the experiment and were housed under conditions of constant temperature and humidity with a 12:12-h light–dark cycle. Rats were allowed to adapt to these conditions for several days before starting any experimental procedures.

RENAL VASCULAR EXPERIMENTS

We utilized the *in vitro* juxtamedullary nephron technique to evaluate the ability of EET analogs to dilate the afferent arteriole. Sprague-Dawley rats were anesthetized with pentobarbital (40 mg/kg body weight i.p.). The right kidney was isolated and after a midline laparotomy, the right renal artery was cannulated through the superior mesenteric artery. The kidney was immediately perfused with a Tyrode's solution containing 6% albumin and a mixture of ʟ-amino acids. After the microdissection procedures were completed, the renal artery perfusion pressure was set to 100 mm Hg. The tissue surface was continuously superfused with a Tyrode's solution containing 1% albumin. After a 20-min equilibration period, an afferent arteriole was chosen for study, and baseline diameter was measured. After the control period, the afferent arteriole was constricted with phenylephrine and the increase in diameter was assessed in response to increasing concentrations of EET analogs (0.01 nM–1 μm). The afferent arteriole diameter changes to EET analogs were monitored for 3 min at each concentration. Steady-state diameter to EET analogs was attained by the end of the second minute, and the average diameter at the third minute was used for statistical analysis.

TELEMETRY BLOOD PRESSURE MEASUREMENT

To accurately detect changes in blood pressure and heart rate, telemetry transmitters (Data Sciences Inc., St. Paul, MN, USA) were implanted in rats 2 weeks prior to the experimental period according to manufacturer's specifications while under sodium pentobarbital anesthesia as previously described (Imig et al., 2005). In brief, a midline incision was used to expose the abdominal aorta that was occluded to allow insertion of the transmitter catheter. The catheter was secured in place with tissue glue and the transmitter body was sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed with staples that were removed 7 days later after the incision had healed. Rats were allowed to recover from surgery and were returned to individual housing.

A baseline arterial pressure was recorded for one week prior to the experimental period. Mean arterial pressure was continuously recorded throughout the experimental period.

ANGIOTENSIN HYPERTENSION

Telemetry transmitters were implanted into male Sprague-Dawley rats (225–275 g) as described. After a week of basal blood pressure recording, osmotic pumps were implanted (s.c.) to deliver angiotensin at a dose of 60 ng/min for 3 weeks. In the first series of experiments starting on the first day of pump implantation the 11,12-ether-EET analog was administered (i.p.) once daily for three weeks and blood pressure was continuously monitored. The next series of experiments were conducted 12 days after pump implantation. EET analogs were administered (i.p.) as a single dose on day 12 and blood pressure was monitored for 48 h.

SPONTANEOUSLY HYPERTENSIVE RATS

Telemetry transmitters were implanted into male spontaneously hypertensive rats (SHR) as described. After the surgical recovery period, baseline mean arterial pressure was recorded for 1 week. In this series of experiments EET analogs were administered (i.p.) once daily for various number of days and blood pressure was continuously monitored. Administration of EET analogs was stopped and blood pressure monitored for 4 days after treatment withdrawal.

DETERMINATION OF NUDSA BY LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONIZATION–MASS SPECTROMETRY

Samples were analyzed by LC–ESI–MS (Agilent 1100 LC/MSD, SL model). Briefly, the samples (5 μl) were separated on a reverse phase C_{18} column (Kromasil, 250 × 2 mm) using water/acetonitrile containing 0.005% acetic acid as a mobile phase at the flow rate of 0.20 mL/min. The mobile phase started at 90% methanol for 2 min, linearly increased to 100% methanol in 10 min, and held for 10 min. Drying gas flow was 12 l/min, drying gas temperature was 350°C, nebulizer pressure was 35 psig, vaporizer temperature was 325°C, capillary voltage was 3000 V, and fragmentor voltage was 120 V. The detection was made in the negative mode. For quantitative measurements, the m/z 440 and 311 were used for NUDSA and [$^2H_8$]-AA, respectively. The concentrations of compounds in the samples were calculated from the ratios of peak areas to internal standard and compared with the standard curves.

STATISTICAL ANALYSIS

All data are presented as mean ± SEM. Mean arterial blood pressure and % afferent arteriolar relaxation data were analyzed using analysis of variance (ANOVA) for repeated measurements. Differences were considered statistically significant with $p < 0.05$ compared to the control. Analyses were performed using GraphPad Prism Version 4.0 software (GraphPad Software Inc, La Jolla, CA, USA).

RESULTS
EET ANALOGS

Experimental studies of EET analogs *in vivo* have been hampered by difficulties with stability and storage. A series of EET analogs was developed and designed to resist esterification, β-oxidation

and/or metabolism by sEH. **Figure 1** depicts the chemical structures of the EET analogs that were synthesized and tested initially for vascular activity.

EET ANALOG VASCULAR ACTIONS

We have previously demonstrated that 11,12-ether-EET-8-ZE increased mesenteric resistance artery and afferent arteriolar diameter to a similar extent as the endogenous metabolite 11,12-EET (Dimitropoulou et al., 2007; Imig et al., 2008). For this study, we assessed the afferent arteriolar diameter response to a series of nine EET analogs and compared them to 11,12-ether-EET-8-ZE. The EET analogs demonstrated a variable ability to dilate afferent arterioles. Afferent arteriolar dilation to the EET analogs became evident at concentration of 1 nM and reached a maximum at 10 µM. Four of the EET analogs dilated the afferent arteriole to a degree similar to 11,12-ether-EET-8-ZE (**Figure 2**). These four EET analogs with activity similar to 11,12-ether-EET-8-ZE as well as an EET analog with lesser vasodilatory activity were then assessed for their ability to lower blood pressure in hypertension rat models.

EET analogs on blood pressure

We initially assessed the ability of 11,12-ether-EET-8-ZE to lower blood pressure in angiotensin and SHR hypertension. **Figure 3**, top panel depicts the effects of 11,12-ether-EET-8-ZE (1 mg/d i.p.) administered once daily for three weeks on blood pressure angiotensin hypertension. 11,12-ether-EET-8-ZE did not alter the progressive increase in blood pressure in response to chronic angiotensin infusion. The result of higher 11,12-ether-EET-8-ZE doses (10 mg/d or 50 mg/d i.p.) administered to SHR is depicted in the bottom panel of **Figure 3**. As with angiotensin hypertension, 11,12-ether-EET-8-ZE did not decrease mean arterial blood pressure when administered to SHR.

Next, we compared the ability of a bolus dose of the EET analogs, NUDSA (1 mg i.p.) and NUSGLY (1 mg i.p.) with excellent dilator activity to an EET analog, EET-NOX-8-glyceride (1 mg i.p.) with lesser dilator activity to lower blood pressure in angiotensin hypertension. NUDSA and EET-NOX-8-glyceride both transiently lowered blood pressure in rats with angiotensin hypertension. Blood pressure in angiotensin hypertensive rats averaged 152 ± 4 mmHg and decreased by 52 mmHg within 2 h after administration of NUDSA, by 46 mmHg in response to NUSGLY, and by 58 mmHg in response to EET-NOX-8-glyceride (**Figure 4**). The decrease in blood pressure to EET-NOX-8-glyceride was very transient and blood pressure increased to hypertensive levels by the third hour. On the other hand, blood pressure remained lower in NUDSA and NUSGLY treated animals throughout the 24-h period and leveled of at 135 ± 6 and 143 ± 5 mmHg, respectively, during the last 8 h. Heart rate averaged 394 ± 7 bpm and did not change in response to NUDSA, NUSGLY, or EET-NOX-8-glyceride administration. These results suggested that EET analogs with excellent dilator activity could be more effective in lowering blood pressure when administered chronically to hypertensive animals.

We then tested five EET analogs for their ability to lower blood pressure in SHR. Comparison of an EET analog with full to an EET analog with intermediate vasodilator activity is depicted in **Figure 5**. The blood pressure response in SHR treated with EET-NOX-8-glyceride (3 mg/d i.p.) or NUSLY (2 mg/d i.p.)
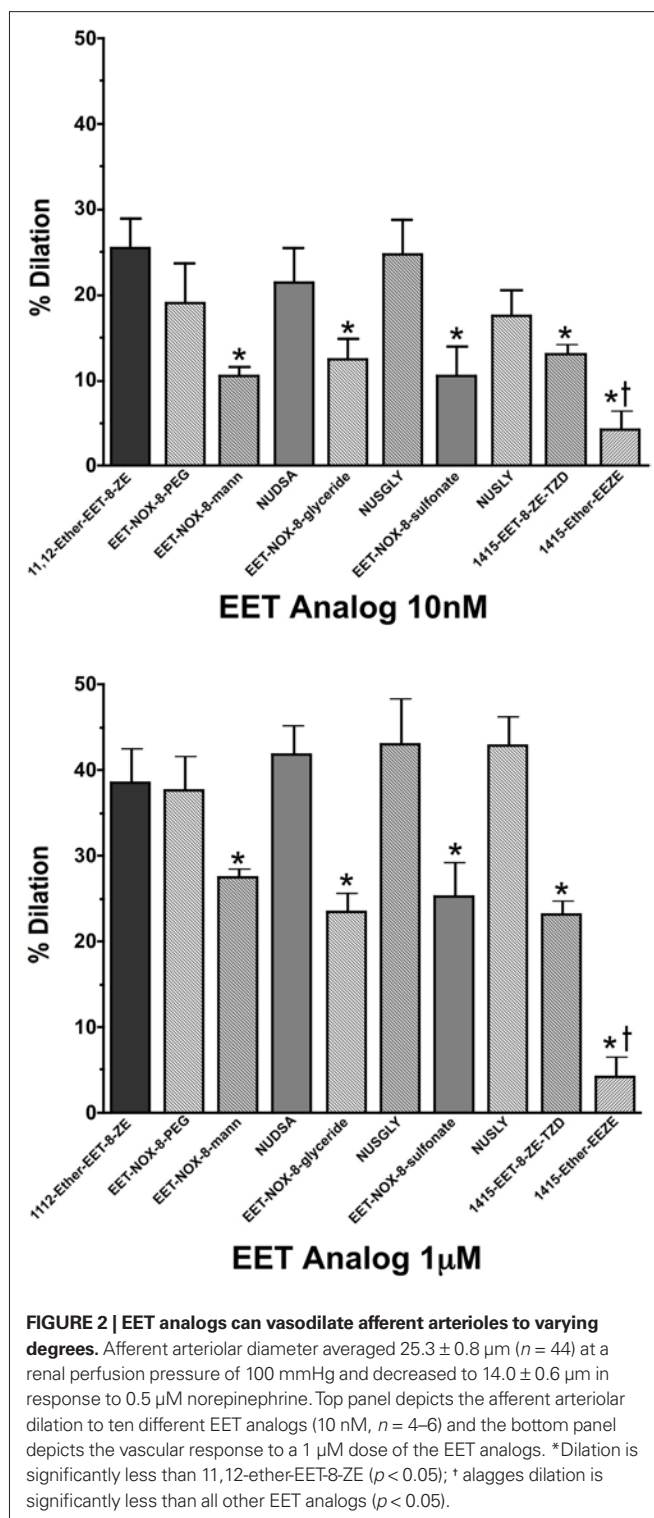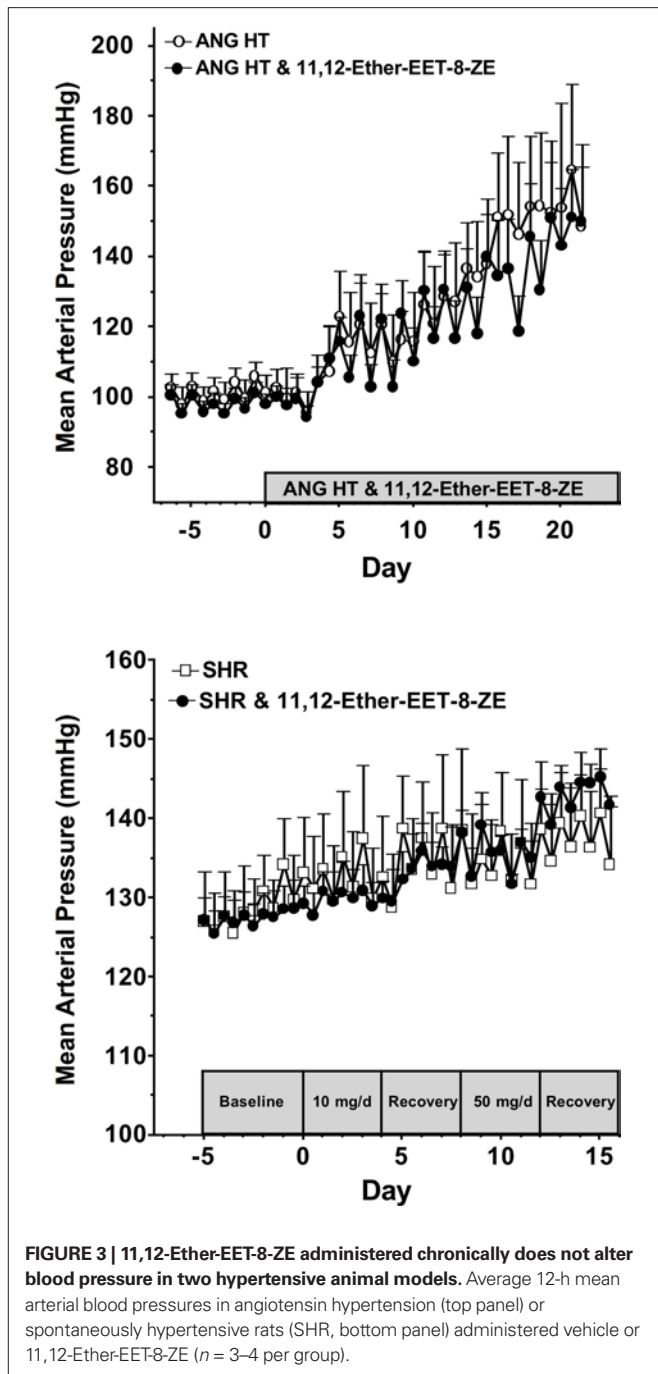

FIGURE 2 | EET analogs can vasodilate afferent arterioles to varying degrees. Afferent arteriolar diameter averaged 25.3 ± 0.8 µm ($n = 44$) at a renal perfusion pressure of 100 mmHg and decreased to 14.0 ± 0.6 µm in response to 0.5 µM norepinephrine. Top panel depicts the afferent arteriolar dilation to ten different EET analogs (10 nM, $n = 4$–6) and the bottom panel depicts the vascular response to a 1 µM dose of the EET analogs. *Dilation is significantly less than 11,12-ether-EET-8-ZE ($p < 0.05$); † alagges dilation is significantly less than all other EET analogs ($p < 0.05$).
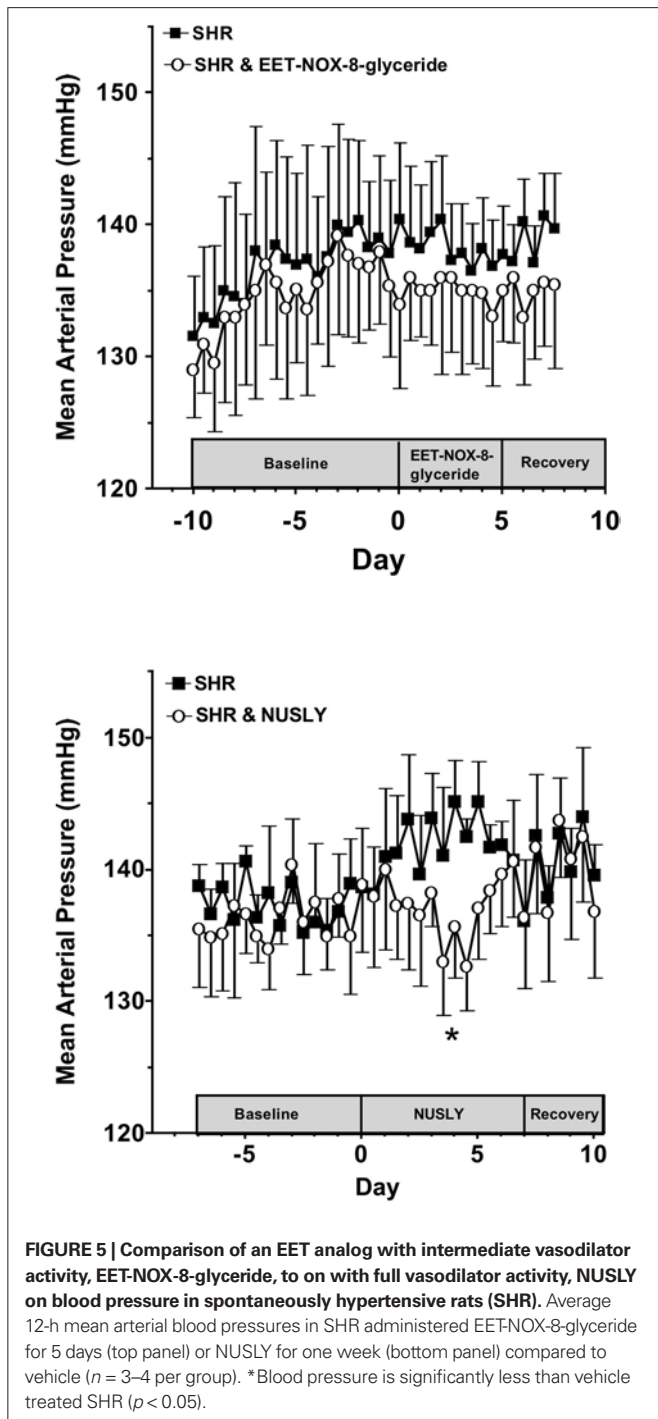
demonstrates that EET analogs with full vasodilator capacity lower blood pressure to a greater extent in SHR. Once daily administration for 5 days with EET-NOX-8-glyceride caused a minimal decrease in blood pressure whereas NUSLY decreased blood pressure by 10 mmHg after 5 days ($p < 0.05$) and this

FIGURE 3 | 11,12-Ether-EET-8-ZE administered chronically does not alter blood pressure in two hypertensive animal models. Average 12-h mean arterial blood pressures in angiotensin hypertension (top panel) or spontaneously hypertensive rats (SHR, bottom panel) administered vehicle or 11,12-Ether-EET-8-ZE ($n = 3–4$ per group).

FIGURE 4 | Blood pressure response to a single bolus injection of EET analogs, NUDSA (top panel), NUSGLY (middle panel), and EET-NOX-8-glyceride (bottom panel). NUDSA and NUSGLY decreased blood pressure over the entire 24-h period whereas EET-NOX-8-glyceride resulted in a transient lowering of blood pressure ($n = 1$ per group).

decrease in blood pressure waned in the last 2 days. The results of two additional EET analogs with excellent dilator activity and an ability to lower blood pressure in the SHR are depicted in **Figure 6**. EET-NOX-8-PEG (2 mg/d i.p.) administered once daily for 10 days to SHR decreased blood pressure 10–15 mmHg ($p < 0.05$, top panel). Once daily administration of NUSGLY (2 mg/d i.p.) for 8 days lowered blood pressure by 15 mmHg in SHR ($p < 0.05$, bottom panel). In each instance blood pressure began to increase in the SHR when the EET analog treatment was discontinued. Taken together, these data suggests that EET
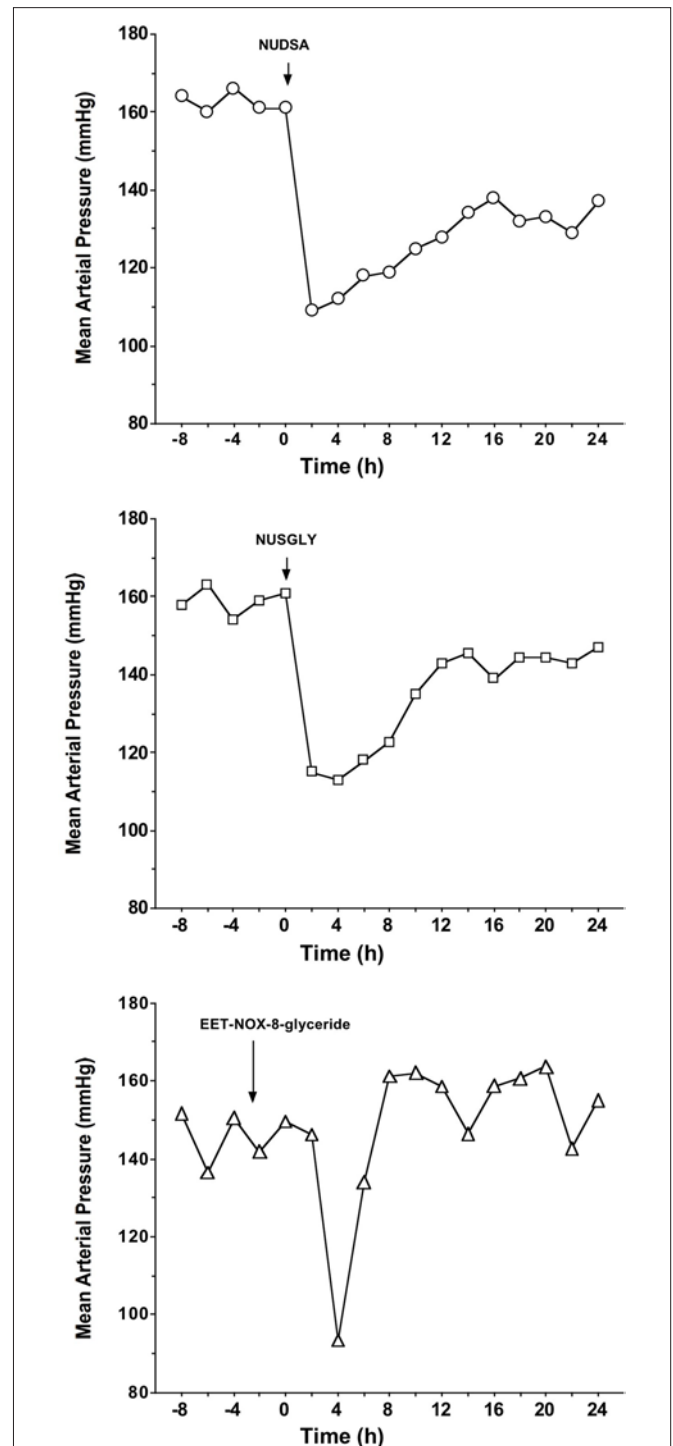
analog dilator activity is just one aspect that can predict *in vivo* blood pressure lowering but other aspects such as bioavailability may limit this biological action.

FIGURE 5 | Comparison of an EET analog with intermediate vasodilator activity, EET-NOX-8-glyceride, to on with full vasodilator activity, NUSLY on blood pressure in spontaneously hypertensive rats (SHR). Average 12-h mean arterial blood pressures in SHR administered EET-NOX-8-glyceride for 5 days (top panel) or NUSLY for one week (bottom panel) compared to vehicle ($n = 3$–4 per group). *Blood pressure is significantly less than vehicle treated SHR ($p < 0.05$).

FIGURE 6 | Two EET analogs administered chronically decrease blood pressure in spontaneously hypertensive rats (SHR). Average 12-h mean arterial blood pressures in SHR administered EET-NOX-8-PEG for ten days (top panel) or NUSGLY for 8 days (bottom panel) compared to vehicle ($n = 3$–4 per group). *Blood pressure is significantly less than vehicle treated SHR ($p < 0.05$).

NUDSA ON BLOOD PRESSURE AND PLASMA LEVELS

Next we tested the effect of NUDSA that had excellent vasodilator activity and has previously been demonstrated to lower blood pressure in a mouse model of metabolic syndrome (Sodhi et al., 2009). Administration of NUDSA (3 mg/d i.p.) for 5 days lowered blood pressure by 10 mmHg in SHR ($p < 0.05$, **Figure 7**, top panel). We then developed an analytical method to measure plasma levels of our lead EET analog, NUDSA that demonstrated *in vivo* blood pressure lowering in angiotensin hypertension and SHR. A quantitative
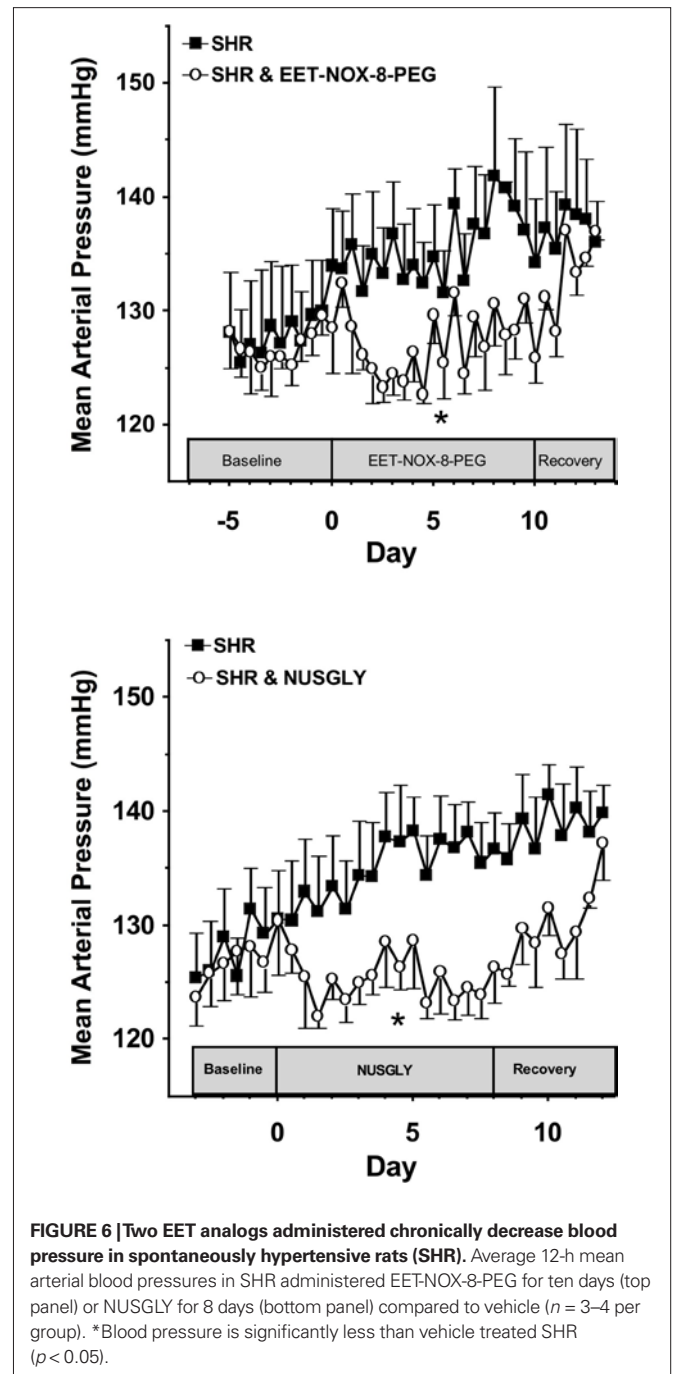
LC/MS method that has a sensitivity to measure 300 pg of NUDSA in a plasma sample. NUDSA administered as a bolus injection resulted in detectable plasma levels for up to 4 h (**Figure 7**, bottom panel). There was also very little evidence for rapid metabolism of NUDSA. These data demonstrate that NUDSA is bioavailable and plasma levels can reach a level that correlates with the anti-hypertensive actions.

DISCUSSION

Epoxyeicosatrienoic acids have cardiovascular actions that implicate them as important contributors to vascular function and blood pressure control (Zeldin, 2001; Spector et al., 2004; Imig, 2005;
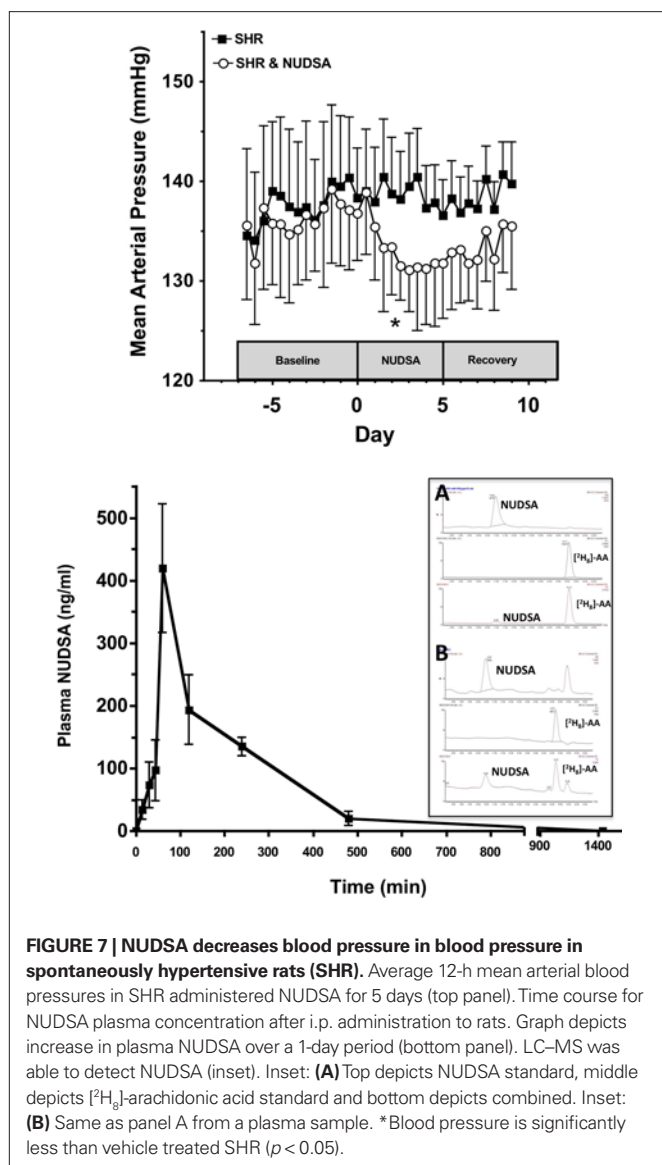
FIGURE 7 | NUDSA decreases blood pressure in blood pressure in spontaneously hypertensive rats (SHR). Average 12-h mean arterial blood pressures in SHR administered NUDSA for 5 days (top panel). Time course for NUDSA plasma concentration after i.p. administration to rats. Graph depicts increase in plasma NUDSA over a 1-day period (bottom panel). LC–MS was able to detect NUDSA (inset). Inset: **(A)** Top depicts NUDSA standard, middle depicts [^{2}H$_8$]-arachidonic acid standard and bottom depicts combined. Inset: **(B)** Same as panel A from a plasma sample. *Blood pressure is significantly less than vehicle treated SHR ($p < 0.05$).

Fleming, 2007). An avenue being explored for potential therapeutic application is the development of EET analogs. EET analogs have the potential to have greater beneficial effects in hypertension since EETs have anti-inflammatory and anti-platelet aggregatory properties (Falck et al., 2003b; Krotz et al., 2004; Imig et al., 2005). We generated a series of 11,12-EET analogs and determined their vascular actions *in vitro* and subsequently tested a subset of this series for their ability to lower blood pressure in hypertension when administered chronically. We found that esterification of the carboxylic group of 11,12-ether-EET-8-ZE with aspartic acid produced the most effective anti-hypertensive EET analog in the series.

Epoxyeicosatrienoic acids analogs of 11,12-EET and 14,15-EET and EET antagonists have been used for many years to examine the cardiovascular actions attributed to EETs (Gauthier et al., 2004; Sudhahar et al., 2010). In particular, analogs of 11,12-EET and 14,15-EET have been used to identify structure activity relationships and cell signaling mechanisms that are responsible for

vasodilation (Falck et al., 2003a; Dimitropoulou et al., 2007; Imig et al., 2008). These studies have determined that an acidic carboxyl group in carbon 1, an Δ^8 double bond, 20-carbon chain length, and a *cis* epoxide are required for full vasodilator activity (Falck et al., 2003a; Dimitropoulou et al., 2007; Imig et al., 2008). We have previously determined that 11,12-ether-EET-8-ZE dilated renal and mesenteric arteries to an extent similar to endogenous 11,12-EET (Dimitropoulou et al., 2007; Imig et al., 2008). A series of analogs were then synthesized and tested for afferent arteriolar dilator activity. Modifications were made to the carboxylic group of 11,12-ether-EET-8-ZE to confer resistance to β-oxidation. 11,12-EET analogs containing an Δ^8 double bond and an ether at the 11,12 position dilated afferent arterioles. Three of these EET analogs were similar to 11,12-ether-EET-8-ZE in respect to vasodilatory capacity. Other modifications to the carboxylic group resulted in EET analogs with an intermediate ability to dilate afferent arterioles. As would be expected, 14,15-ether-EEZE that lacks an Δ^8 double bond failed to dilate the afferent arteriole. These findings determined that modifications to the carboxylic group of 11,12-ether-EET-8-ZE result in EET analogs that have similar or slightly diminished vasodilatory activity.

Even though EET analogs have been used in cell culture and *in vitro* systems for many years, there has been limited success with *in vivo* administration in either the acute or chronic setting. 11,12-Ether-EET-8-ZE did not alter blood pressure in angiotensin hypertension or SHR when administered chronically for a period of days and weeks. Since 11,12-ether-EET-8-ZE contains a carboxylic group that is susceptible to esterification and β-oxidation, we developed another series of EET analogs. In this study we determined that modification of the carboxylic group to resist β-oxidation resulted in EET analogs that decreased blood pressure in hypertension. Interestingly, EET-NOX-8-glyceride that had intermediate vasodilatory activity transiently decreased blood pressure in angiotensin hypertension and did not significantly lower blood pressure in the SHR when administered over a 5-day period. On the other hand, NUDSA that vasodilated the afferent arteriole similar to 11,12-ether-EET-8-ZE decreased blood pressure in angiotensin hypertension over a 24-h period and decreased blood pressure over a 5-day period in SHR. These decreases in blood pressure were not accompanied by a change in heart rate. A recent study determined the ability of NUDSA to ameliorate the metabolic syndrome phenotype in heme-oxygenase 2 null mice (Sodhi et al., 2009). NUDSA decreased blood pressure and prevented obesity-associated vascular and kidney damage in metabolic syndrome mice (Sodhi et al., 2009). This study and the results of the current study demonstrate a first step toward the therapeutic application of EET analogs for cardiovascular diseases.

One aspect that was not determined in the experimental study where mice were chronically administered NUDSA was bioavailability. We developed an LC–MS method to determine NUDSA levels in rat plasma. An injection of NUDSA was given and plasma levels determined at different time points. Plasma levels of NUDSA peaked 2 h after administration and were significantly lower at 8 h. We did not determine clearance, metabolism, or tissue NUDSA levels in the current study. A more thorough pharmacokinetic analysis would be necessary if this or another EET analog was developed for preclinical trials.

In conclusion, we developed a series of EET analogs and evaluated their ability to dilate afferent arterioles and lower blood pressure in angiotensin hypertension and SHR. Amidation of the 11,12-ether-EET-8-ZE carboxylic group with aspartic acid resulted in a promising EET analog that possessed vasodilatory activity and lowered blood pressure in hypertension. These experimental findings provide evidence for the possible future use of EET analogs for the treatment of cardiovascular diseases.

REFERENCES

Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.* 78, 415–423.

Dimitropoulou, C., West, L., Field, M. B., White, R. E., Reddy, L. M., Falck, J. R., and Imig, J. D. (2007). Protein phosphatase 2A and Ca^{2+}-activated K + channels contribute to 11,12-epoxyeicosatrienoic acid analog mediated mesenteric arterial relaxation. *Prostaglandins Other Lipid Mediat.* 83, 50–61.

Falck, J. R., Kodela, R., Manne, R., Atcha, R., Puli, N., Dubasi, N., Manthati, V. L., Capdevila, J. H., Yi, X. Y., Goldman, D. H., Morisseau, C., Hammock, B. D., and Campbell, W. B. (2009). 14,15-Epoxyeicosa-5,8,11-trienoic acid (14,15-EET) surrogates containing epoxide bioisosteres: influence upon vascular relaxation and soluble epoxide hydrolase inhibition. *J. Med. Chem.* 52, 5069–5075.

Falck, J. R., Krishna, U. M., Reddy, Y. K., Kumar, P. S., Reddy, K. M., Hittner, S. B., Deeter, C., Sharma, K. K., Gauthier, K. M., and Campbell, W. B. (2003a). Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation. *Am. J. Physiol. Heart Circ. Physiol.* 284, H337–H349.

Falck, J. R., Reddy, L. M., Reddy, Y. K., Bondlela, M., Krishna, U. M., Ji, Y., Sun, J., and Liao, J. K. (2003b). 11,12-epoxyeicosatrienoic acid (11,12-EET): structural determinants for inhibition of TNF-alpha-induced VCAM-1 expression. *Bioorg. Med. Chem. Lett.* 13, 4011–4014.

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., and Busse, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 401, 493–497.

Fleming, I. (2007). Epoxyeicosatrienoic acids, cell signaling and angiogenesis. *Prostaglandins Other Lipid Mediat.* 82, 60–67.

Gauthier, K. M., Falck, J. R., Reddy, L. M., and Campbell, W. B. (2004). 14,15-EET analogs: characterization of structural requirements for agonist and antagonist activity in bovine coronary arteries. *Pharmacol. Res.* 49, 515–524.

Gross, G. J., Gauthier, K. M., Moore, J., Falck, J. R., Hammock, B. D., Campbell, W. B., and Nithipatikom, K. (2008). Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection produced by exogenous or endogenous EETs in the canine heart. *Am. J. Physiol. Heart Circ. Physiol.* 294, H2838–H2844.

Hercule, H. C., Schunck, W. H., Gross, V., Seringer, J., Leung, F. P., Weldon, S. M., da Costa Gonclalves, A. C. H., Huand, Y., Luft, F. C., and Gollasch, M. (2009). Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Aterioscler. Thromb. Vasc. Biol.* 29, 54–60.

Imig, J. D. (2005). Epoxide hydrolase and epoxygenase metabolites as therapeutic targets for renal diseases. *Am. J. Physiol. Renal Physiol.* 289, F496–F503.

Imig, J. D., Dimitropoulou, C., Reddy, D. S., White, R. E., and Falck, J. R. (2008). Afferent arteriolar dilation to 11,12-EET analogs involves PP2A activity and Ca2 + -activated K + Channels. *Microcirculation* 15, 137–150.

Imig, J. D., and Falck, J. R. (2008a). Compositions and methods for the treatment of renal and cardiovascular disease. U.S. Pat. Appl. Publ., Cont.-in-part of U.S. Ser. No. 866,395. US 2008153889 A1 20080626 CAN 149:104528 AN 2008:773879.

Imig, J. D., and Falck, J. R. (2008b). 11-Nonyloxyundec-8(Z)-enoic acid and derivatives for the treatment of renal and cardiovascular disease. U.S. Pat. Appl. Publ. US 2008146663 A1 20080619 CAN 149:70453 AN 2008:743378.

Imig, J. D., and Falck, J. R. (2009). Compositions and methods for the treatment of renal and cardiovascular disease. US Patent #7,550,617.

Imig, J. D., Inscho, E. W., Deichmann, P. C., Reddy, K. M., and Falck, J. R. (1999). Afferent arteriolar vasodilation to the sulfonimide analog of 11,12-epoxyeicosatrienoic acid involves protein kinase A. *Hypertension* 33, 408–413.

Imig, J. D., Zhao, X., Falck, J. R., Wei, S., and Capdevila, J. H. (2001). Enhanced renal microvascular reactivity to angiotensin II in hypertension is ameliorated by the sulfonimide analog of 11,12-epoxyeicosatrienoic acid. *J. Hypertens.* 19, 983–992.

Imig, J. D., Zhao, X., Zaharis, C. Z., Olearczyk, J. J., Pollock, D. M., Newman, J. W., Kim, I. H., Watanabe, T., and Hammock, B. D. (2005). An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. *Hypertension* 46, 975–981.

Krotz, F., Riexinger, T., Buerkle, M. A., Nithipatikom, K., Gloe, T., Sohn, H. Y., Campbell, W. B., and Pohl, U. (2004). Membrane-potential-dependent inhibition of platelet adhesion to endothelial cells by epoxyeicosatrienoic acids. *Arterioscler. Thromb. Vasc. Biol.* 24, 595–600.

Node, K., Huo, Y., Ruan, X., Yang, B., Spiecker, M., Ley, K., Zeldin, D. C., and Liao, J. K. (1999). Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 285, 1276–1279.

Potente, M., Fisslthaler, B., Busse, R., and Fleming, I. (2003). 11,12-Epoxyeicosatrienoic acid-induced inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1. *J. Biol. Chem.* 278, 29619–29625.

Seubert, J. M., Zeldin, D. C., Nithipatikom, K., and Gross, G. J. (2007). Role of epoxyeicosatrienoic acids protecting the myocardium following ischemia / reperfusion injury. *Prostaglandins Other Lipid Mediat.* 82, 50–59.

Sodhi, K., Inoue, K., Gotlinger, K. H., Canestraro, M., Vanella, L., Kim, D. H., Manthati, V. L., Koduru, S. R., Falck, J. R., Schwarzman, M. L., and Abraham, N. G. (2009). Epoxyeicosatrienoic acid agonist rescues metabolic syndrome phenotype of HO-2-null mice. *J. Pharmacol. Exp. Ther.* 331, 906–916.

Spector, A. A., Fang, X., Snyder, G. D., and Weintraub, N. L. (2004). Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function. *Prog. Lipid Res.* 43, 55–90.

Sudhahar, V., Shaw, S., and Imig, J. D. (2010). Epoxyeicosatrienoic acid analogs and vascular function. *Curr. Med. Chem.* 17, 1181–1190.

Sun, J., Sui, X., Bradbury, J. A., Zeldin, D. C., Conte, M. S., and Liao, J. K. (2002). Inhibition of vascular smooth muscle

cell migration by cytochrome p450 epoxygenase-derived eicosanoids. *Circ. Res.* 90, 1020–1027.

Yang, Q., Zhang, R. Z., Yim, A. P., and He, G. W. (2003). Effect of 11,12-epoxyeicosatrienoic acid as an additive to St. Thomas' cardioplegia and University of Wisconsin solutions on endothelium-derived hyperpolarizing factor-mediated function in coronary microarteries: influence of temperature and time. *Ann. Thorac. Surg.* 76, 1623–1630.

Yang, W., Holmes, B. B., Gopal, V. R., Kishore, R. V., Sangras, B., Yi, X. Y., Falck, J. R., and Campbell, W. B. (2007). Characterization of 14,15-epoxyeicosatrienoyl-sulfonamides as 14,15-epoxyeicosatrienoic acid agonists: use for studies of metabolism and ligand binding. *J. Pharmacol. Exp. Ther.* 321, 1023–1031.

Zeldin, D. C. (2001). Epoxygenase pathways of arachidonic acid metabolism. *J. Biol. Chem.* 276, 36059–36062.

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